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14. ABSTRACT Cdk4 is an important regulator of G1/S cell cycle progression in mammalian cells. In humans, the Cdk4 gene is amplified in 16% of sporadic breast tumors. In mice, the loss of Cdk4 affects the development of the mammary glands. Our studies to determine the role of Cdk4 in Neu, Wnt-1, and Ras-induced breast tumorigenesis indicated that the absence of Cdk4 impairs Neu and Ras-induced mammary tumorigenesis but not that induced by Wnt-1. Specifically, while the tumor incidences in Cdk4-null MMTV-Ras and MMTV-Neu mice were dramatically reduced when compared to their respective wild-type transgenic counterparts (0% versus 70% and 14% versus 97%, respectively), the loss of Cdk4 did not affect the tumor incidence in the MMTV-Wnt-1 mouse model. In addition to Cdk4 null models, we also assessed the role of the Cdk4R24C mutation played in mammary tumorigenesis. Interestingly, the onset of tumors is significantly delayed in MMTV-Ras transgenic mice that express the hyperactive Cdk4R24C mutated allele when compared to those mice that express wild-type Cdk4. Analysis of the tumors and normal tissues suggests that the Cdk4 gene may play a role in modulating oncogenic stress-induced DNA damage checkpoint responses.					
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## Table of Contents

Introduction.....	4
Body.....	4
Key Research Accomplishments.....	11
Reportable Outcomes.....	11
Conclusions.....	12
References.....	12
Appendices.....	14

## Introduction:

Breast cancer has second highest incidence of all types of cancer among women. Many proteins are deregulated in breast cancer, including cell cycle regulatory proteins (1). The gene encoding one of these proteins, *Cyclin dependent kinase 4 (Cdk4)*, is amplified in 16% of sporadic breast tumors and this amplification correlates with high Cdk4 protein levels (2). Mutations of Cdk4, especially the hyperactive R24C mutation, have also been identified in human melanomas (3,4). In mice, the Cdk4 (R24C) mutation leads to the development of tumors in multiple tissues including mammary tissue (5,6). Cdk4, in association with Cyclin D functions during the progression of G1 phase of the cell cycle. Approximately 50% of human mammary carcinomas express abnormally high levels of Cyclin D1 (7,8,9,10,11), and these levels are maintained throughout all stages of the disease (i.e., from in situ carcinoma to invasive carcinomas) (10,12,13). In mice, the transgenic expression of Cyclin D1 results in mammary adenocarcinomas, which indicates the oncogenic role of Cyclin D1 in mammary epithelium (14). Further, the loss of *Cyclin D1* in mice results in resistance to breast cancers induced by *neu* and *ras* oncogenes but not to those induced by *c-Myc* or *Wnt-1* (15). Taken together, these results suggest that the activity of D-type cyclin/Cdk4 complexes is required for fibroblast transformation. However, it has also been suggested that the oncogenic function of cyclin D1 is independent of its ability to activate Cdks and is perhaps linked to the direct effects of cyclin D1 in controlling the expression of a subset of genes that are co-up-regulated in human tumors with deregulated cyclin D1 (16). The results of this study illustrate the importance of Cdk4 in oncogene-induced breast tumorigenesis.

## Body:

### 1. Effect of loss of Cdk4 expression on *neu*- and *wnt*-mediated breast tumorigenesis.

To study the role of Cdk4 in *neu*- and *wnt*-induced breast tumorigenesis, *Cdk4(neo/neo)* mice were bred with MMTV-*neu* and MMTV-*wnt* transgenic mice to generate *Cdk4(neo/neo):MMTV-neu*, and *Cdk4(neo/neo):MMTV-wnt* mice, respectively. Whole-mount and histopathologic sections of the mammary glands derived from virgin adult mice (~14 weeks) from these different crosses (Fig. 2B, C, D and E of appended article) showed that *Cdk4(+/+):MMTV-neu* mice exhibit proliferative disturbances in the mammary epithelium as evidenced by the appearance of multiple hyperplastic and dysplastic nodules that infiltrate the mammary fat pad (Fig. 2B and F of appended article). Similar examination of whole-mount and histopathologic sections of mammary tissue derived from *Cdk4(neo/neo):MMTV-neu* mice showed that the ductal outgrowth and branching morphogenesis was considerably reduced compared with *Cdk4(+/+):MMTV-neu* mice with distinctive absence of any hyperplastic or dysplastic nodules that are characteristic of the later group of mice (Fig. 2C and G of appended article). Histopathologic examination of these mammary glands also failed to show abnormal proliferative disturbances in the mammary epithelium of *Cdk4(neo/neo):MMTV-neu* mice (Fig. 2C and G of appended article). This does not seem

to be due to lack of Neu expression, as equal levels of Neu protein was seen in both *Cdk4(+/+):MMTV-neu* and *Cdk4(neo/neo):MMTV-neu* mice (Fig. 2J of appended article). These results suggest that Cdk4 expression is essential for the appearance of MMTV-*neu*–induced proliferative disturbances that are seen in *Cdk4(+/+):MMTV-neu* mice.

In contrast to MMTV-*neu* mice, the mammary glands of virgin *Cdk4(+/+):MMTV-wnt* mice showed precocious lobuloalveolar development that resembles that of *Cdk4(+/+)* pregnant female mice (Fig. 2D and H of appended article). Histopathologic examination of these mammary glands revealed extensive appearance of hyperplastic alveolar nodules, which seem to be preneoplastic lesions. Similar examination of whole-mount and histopathologic sections of mammary tissue derived from *Cdk4(neo/neo):MMTV-wnt* mice showed that the ductal outgrowth and branching morphogenesis was unaltered compared with *Cdk4(+/+):MMTV-wnt* mice (Fig. 2E and I of appended publication). Histopathologic examination of these mammary tissues again showed extensive appearance of hyperplastic alveolar nodules, similar to that seen with wild-type MMTV-*wnt* mice. These results indicate that MMTV-*wnt*–induced proliferative disturbances do not require Cdk4 expression.

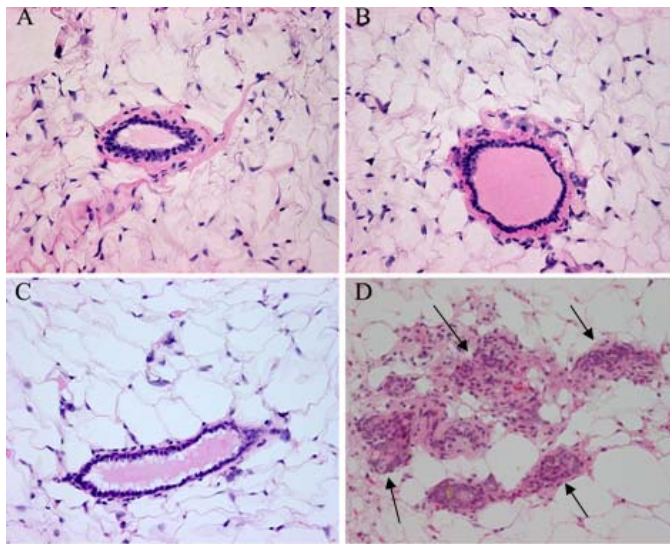
## **2. Loss of expression of Cdk4 influences the incidence of mammary carcinomas.**

To determine whether Cdk4 plays a similar role in the development of breast carcinomas, we monitored the four groups of transgenic mice for the appearance of breast tumors. The results of this study (Fig. 3A of appended publication) show that ~97% of the *Cdk4(+/+):MMTV-neu* mice develop breast cancer between 28 to 75 weeks of age. The rest of the mice were found to develop salivary gland tumors. In sharp contrast, only ~14% of the *Cdk4(neo/neo):MMTV-neu* mice develop signs of breast cancer and this incidence was found to occur only after ~60 weeks of age; when these tumors arise, they were very small in size compared with their wild-type counterparts. Calculation of *P* values showed a highly significant increase in tumor frequency ( $P = 2.3 \times 10^{-6}$ ) for *Cdk4(+/+):MMTV-neu* mice as opposed to their *neo/neo* counterparts. These observations suggest that development of breast tumors in MMTV-*neu* transgenic mice requires normal expression of Cdk4.

In contrast to *Cdk4(+/+):MMTV-neu* mice, both *Cdk4(+/+):MMTV-wnt* mice and *Cdk4(neo/neo):MMTV-wnt* mice exhibited a rapid onset of breast tumors around 10 weeks of age and >90% of these mice developed breast tumors by the age of 30 weeks (Fig. 3B of appended publication). Our studies also show that there was a slight delay in the development of breast tumors in *Cdk4(neo/neo):MMTV-wnt* mice compared with their wild-type counterparts. In contrast to the results observed for MMTV-*neu* mice, no significant difference in tumor frequency ( $P = 0.7264$ ) was observed between *Cdk4(+/+):MMTV-wnt-1* and their *neo/neo* counterparts. The incidence of breast tumors was seen in both male and female mice. These observations show that Cdk4 expression is dispensable for MMTV-*wnt*–induced breast tumorigenesis.

The histopathologic sections of the tumors is shown in Fig. 3C of the appended publication. These sections show that *Cdk4*(+/+):MMTV-*neu* tumors have a high density of epithelial cells, whereas the tumor sections of *Cdk4*(*neo/neo*):MMTV-*neu* mice show increased infiltration by connective tissue. MMTV-*wnt* tumors in a *Cdk4*(+/+) or *Cdk4*(*neo/neo*) background showed a similar phenotype with a high density of epithelial cells. Interestingly, these tumors show increased vasculature, suggesting that the Wnt pathway promotes angiogenesis, which might explain the very rapid growth of tumors in these mice.

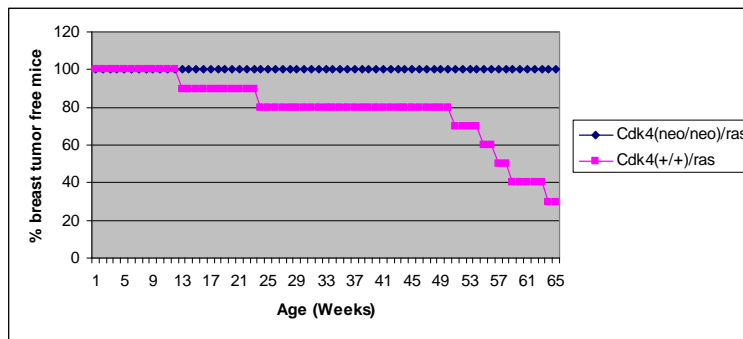
**3. Importance of Cdk4 in v-Ha-ras-induced mammary tumorigenesis:** To gain an understanding of the role of Cdk4 in Ras-induced breast tumorigenesis, *Cdk4*(+/+*neo*) mice were bred with MMTV-v-Ha-ras transgenic mice to generate *Cdk4*(*neo/neo*):MMTV-v-Ha-ras mice and *Cdk4*(+/+):MMTV-v-Ha-ras mice. The histopathological sections of the mammary glands derived from virgin adult mice (approximately 14 weeks old mice) from these crosses showed that *Cdk4*(+/+):MMTV-v-Ha-ras mice exhibit proliferative disturbances in the mammary epithelium as evidenced by the appearance of multiple hyperplastic and dysplastic changes that resulted in the loss of ductal architecture (Figure 3.1). Similar examination of the histopathological sections of mammary tissue derived from *Cdk4*(*neo/neo*):MMTV-v-Ha-ras mice revealed a well defined ductal architecture with very little or complete absence of any hyperplastic and dysplastic changes. These results suggest that Cdk4 expression is essential for the appearance of MMTV-v-Ha-ras-induced proliferative disturbances.



**Figure 3.1 Loss of Cdk4 abrogates Ras-induced hyperplastic and dysplastic changes in the epithelial tissue of mammary glands.** Formalin fixed paraffin embedded mammary gland sections of *Cdk4*(*neo/neo*) (A), *Cdk4*(+/+) (B), *Cdk4*(*neo/neo*): MMTV-v-Ha-ras (C), and *Cdk4*(+/+): MMTV-v-Ha-ras (D) mice were deparaffinized and stained with H&E stain (40x). Arrows in D indicate the loss of ductal architecture.

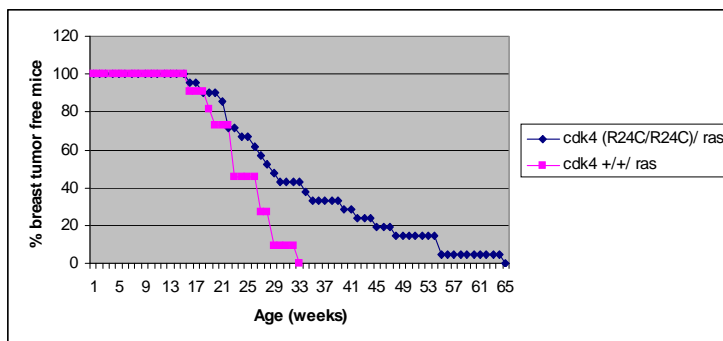
The tumorigenesis studies of these mice presented in Figure 3.2 show that approximately 70% of *Cdk4*(+/+):MMTV-v-Ha-ras mice develop mammary tumors between the age of 12 to 64 weeks. In contrast, none of the *Cdk4*(*neo/neo*):MMTV-v-Ha-ras mice showed any signs of tumor development and remained tumor-free beyond the age of 65 weeks. These observations suggest that the development of mammary tumors in MMTV-v-Ha-

*ras* transgenic mice requires normal expression of Cdk4 which is in accordance with the requirement of Cdk4 for Ras-dependent skin tumor development (17).



**Figure 3.2 Cdk4 is required for v-Ha-Ras-induced breast tumorigenesis.** H-Ras transgenic mice on *Cdk4(neo/neo)* and *Cdk4(+/+)* backgrounds were monitored for tumor formation for a period of 65 weeks.

**4. Effect of the Cdk4R24C mutation on Ras-induced breast Tumorigenesis:** Mouse modeling experiments have shown that mice harboring a knock-in *Cdk4R24C* mutation develop tumors at an accelerated rate (6). Furthermore, these studies revealed that *Cdk4(R24C/R24C)* mice exhibit an increased susceptibility to carcinogen-induced tumorigenesis (18,6). Because some of the carcinogens such as DMBA used in these studies are known to mediate their oncogenic function by targeting the *ras* gene, it was of interest to determine whether the Cdk4R24C mutation promotes Ras-induced tumorigenesis in mammary tissue. To address this question, MMTV-v-Ha-*ras* transgenic mice were bred either with *Cdk4(R24C/R24C)* or *Cdk4(+/+)* mice to generate *Cdk4(R24C/R24C):MMTV-v-Ha-ras* and *Cdk4(+/+):MMTV-v-Ha-ras* mice. The onset and progression of mammary tumors were monitored over a period of 65 weeks. The results of this study presented in Figure 4.1 show that while 100% of the *Cdk4(+/+):MMTV-v-Ha-ras* mice developed mammary tumors in about 33 weeks, the *Cdk4*

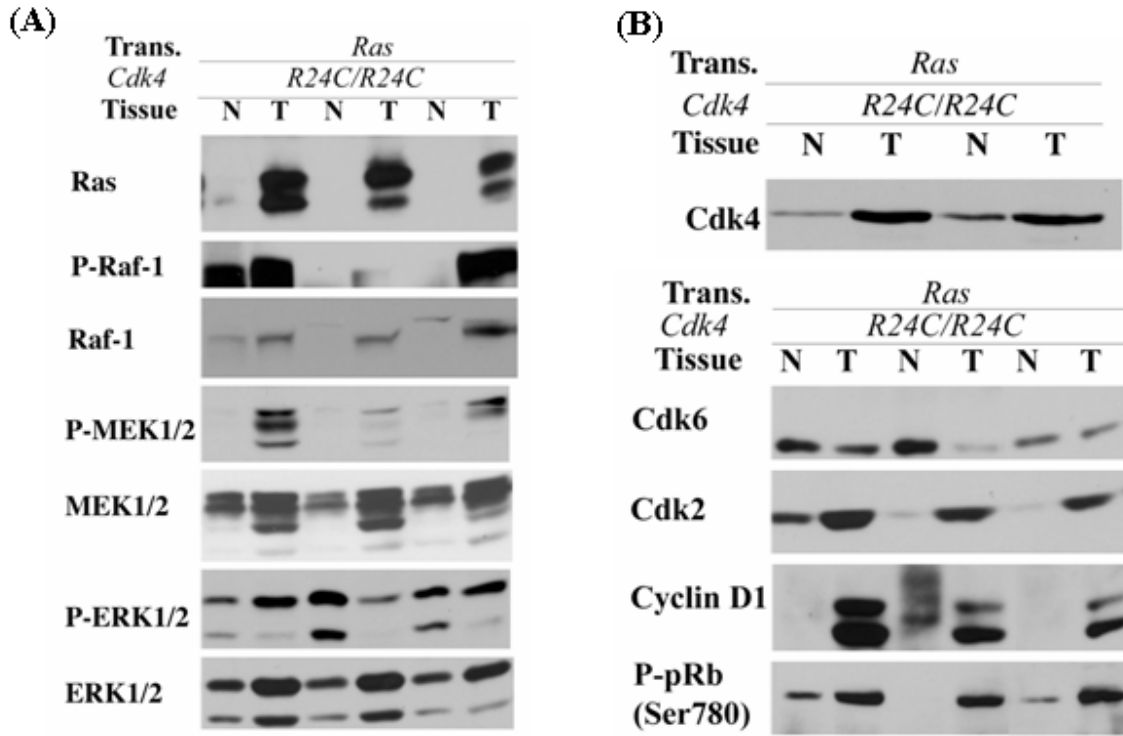


**Figure 4.1 R24C mutation of Cdk4 does not accelerate Ras-induced breast tumorigenesis.** *Cdk4(R24C/R24C):MMTV-v-Ha-ras* and *Cdk4(+/+):MMTV-v-Ha-ras* mice were examined for Ras-induced breast tumor incidence over a period of 65 weeks.

(*R24C/R24C*): MMTV-v-Ha-*ras* mice developed tumors in over a 65 week period, indicating a lag in the tumor development of these mice. This result shows that the R24C mutation retards the development of Ras-induced mammary tumors and is in contrast to previous studies (6) which have shown that this mutation accelerates tumorigenesis.

To gain an understanding of the molecular mechanisms associated with the delayed tumorigenesis seen in *Cdk4(R24C/R24C):MMTV-v-Ha-ras* mice, the expression of

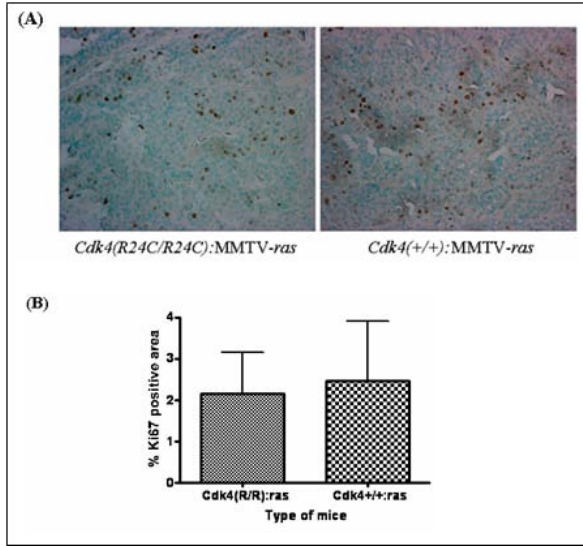
proteins that function in the Ras pathway and as modulators of the cell cycle were analyzed by Western blot analysis. As represented in figure 4.2A, while all of the proteins examined were expressed at similar levels in both the normal and tumor samples, increased levels of phosphorylated MEK1/2 were observed in the tumors. Similarly, the tumors also expressed higher levels of Cdk2 and cyclin D1.



**Figure. 4.2 Analysis of Ras pathway and cell cycle proteins.** Protein extracted from normal (N) and mammary tumor tissues (T) of Cdk4 (R24C/R24C):MMTV-v-Ha-ras mice was subjected to Western blot analysis using antibodies directed against (A) Ras, Phospho-Raf1 (P-Raf1), Raf-1, Phospho-MEK1/2 (P-MEK1/2), MEK1/2, Phospho ERK1/2 (P-ERK1/2) and ERK1/2 proteins and (B) Cdk4, Cdk6, Cdk2, Cyclin D1 and P-pRb (Ser780).

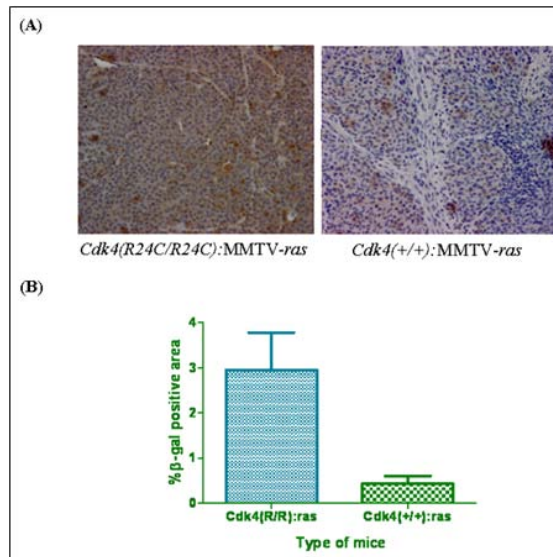
We also assessed the expression of proliferation and senescence markers using immunohistochemical analysis. The results of this study, shown in Figure 4.3A & B, reveal the presence of Ki67 positive cells (Nuclei stained dark brown) in the tumor tissues of both *Cdk4*(+/+):MMTV-v-Ha-ras and *Cdk4*(R24C/R24C):MMTV-v-Ha-ras mice. The quantification of these Ki67 positive areas using Bioquant software indicated no significant difference in the proliferation index of these tumors, suggesting that the lag in tumor development is not due to inadequate cell proliferation.





**Figure 4.3 Analysis of proliferation marker Ki67 in the tumor sections of *Cdk4(+/+): MMTV-v-Ha-ras* and *Cdk4 (R24C/R24C): MMTV-v-Ha-ras* mice.** (A) Formalin fixed paraffin embedded normal and tumor sections of the mammary glands of *Cdk4(+/+): MMTV-v-Ha-ras* and *Cdk4(R24C/R24C): MMTV-v-Ha-ras* mice were subjected to immunohistochemical analysis using a Ki67 antibody (40x). (B) Quantitation of Ki67 expression was determined using Bioquant software and analyzed using unpaired *t* test provided in Prism software.

Over-expression of Ras has been shown to induce a permanent growth arrest in cultured primary cells in the absence of cooperating oncogenic mutations (19,20). Studies have also indicated that high levels of Ras induces irreversible cellular senescence (21). To determine whether induction of senescence plays a role in the delayed tumor progression seen in *Cdk4(R24C/R24C): MMTV-v-Ha-ras* mice, we analyzed tumor tissues derived from *Cdk4(+/+):MMTV-v-Ha-ras* and *Cdk4(R24C/R24C):MMTV-v-Ha-ras* mice for the expression of  $\beta$ -galactosidase, a marker for senescence, using immunohistochemistry. The results of this study presented in Figure 4.4A show elevated expression of  $\beta$ -galactosidase, a marker of senescence, in the breast tumor sections of



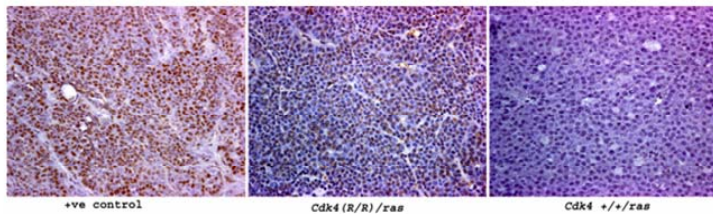
**Figure 4.4 Increased senescence in the mammary tumor tissues of *Cdk4(R24C/R24C):MMTV-v-Ha-ras* mice when compared to *Cdk4(+/+): MMTV-v-Ha-ras* mice.** (A) Formalin fixed paraffin embedded normal and tumor sections of the mammary glands of *Cdk4(+/+): MMTV-v-Ha-ras* and *Cdk4 (R24C/R24C):MMTV-v-Ha-ras* mice were subjected to immunohistochemical analysis using an antibody directed against the  $\beta$ -galactosidase protein (40x). (B) Quantitation of  $\beta$ -galactosidase expression was determined using Bioquant software and analyzed using unpaired *t* test provided in Prism software.

*Cdk4(R24C/R24C):MMTV-v-Ha-ras* mice when compared to their wild-type transgenic counterparts. Computer-assisted quantitation of the immunohistochemical staining using Bioquant software further confirmed a significant increase in the levels of  $\beta$ -galactosidase positive areas in the tumor sections of *cdk4(R24C/R24C):MMTV-v-Ha-ras*

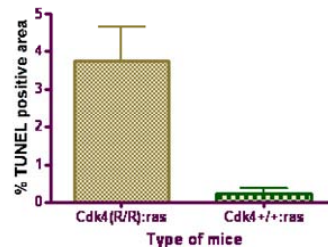
mice (Figure 4.4B). These results suggest that co-expression of mutant Ras and Cdk4R24C proteins in mammary epithelial tissue results in an increased activation of senescence pathways that could explain the lag seen in the development of mammary tumors in *Cdk4(R24C/R24C):MMTV-v-Ha-ras* mice.

To determine whether co-expression of activated Ras and Cdk4 proteins results in increased apoptotic death of mammary epithelial cells, we performed TUNEL staining to identify apoptotic cells in tumor tissue sections derived from *Cdk4(+/+):MMTV-v-Ha-ras* and *Cdk4(R24C/R24C):MMTV-v-Ha-ras* mice. The results from these studies presented in figure 4.5 show a significant increase in TUNEL-positive cells in the tumors of *Cdk4(R24C/R24C):MMTV-v-Ha-ras* mice when compared to their wild-type transgenic counterparts (Figure 4.5B).

(A)



(B)

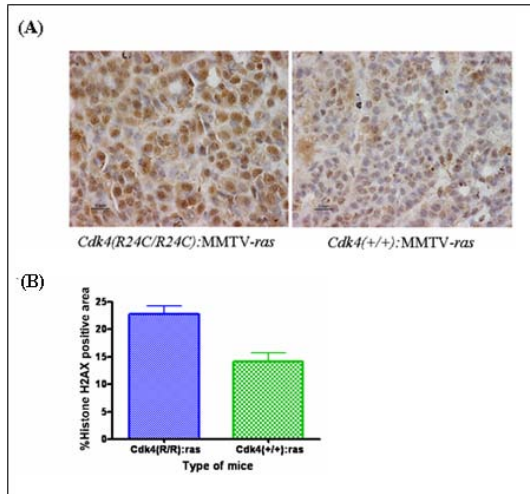


**Figure 4.5 Mammary tumors of *Cdk4 (R24C/R24C):MMTV-v-Ha-ras* mice exhibit enhanced apoptosis when compared to *Cdk4 (+/+): MMTV-v-Ha-ras* mice.** (A) TUNEL analysis of formalin fixed paraffin embedded normal and tumor sections of the mammary glands of *Cdk4 (+/+): MMTV-v-Ha-ras* and *Cdk4 (R24C/R24C):MMTV-v-Ha-ras* mice (40x). (B) Quantitation of TUNEL-positive cells was determined using Bioquant software and analyzed using unpaired *t* test provided in Prism software.

The Ras oncoprotein has previously been shown to induce senescence through activation of the p38MAPK-MKK3/6 (22) pathway or via activation of the DNA-damage checkpoint response (DDR) (23, 24, 25). Ras has also been shown to induce apoptosis through the JNK pathway (26), which in turn leads to apoptosis or cell cycle arrest. Our analysis of the p38MAPK-MKK3/6 pathway did not show an increase in the activity of p38MAPK or JNK in tumor tissues derived from *Cdk4(+/+):MMTV-v-Ha-ras* and *Cdk4(R24C/R24C):MMTV-v-Ha-ras* mice (data not shown) suggesting that activation of p38 MAPK is not responsible for the observed differences in senescence.

To determine whether the DNA-damage checkpoint response (DDR) is the major cause for the enhanced senescence and apoptosis seen in the mammary tumor tissues of *Cdk4(R24C/R24C):MMTV-v-Ha-ras* mice, we examined the expression of  $\gamma$ -H2AX, a marker that is commonly used to monitor double strand DNA breaks, using immunohistochemical analysis (27,28). Our analysis of tumor tissue sections revealed a significant increase in the appearance of subnuclear foci containing  $\gamma$ -H2AX protein in

tissues derived from *Cdk4(R24C/R24C):MMTV-v-Ha-ras* mice compared to their wild-type transgenic counterparts (Figure 4.6). These results suggest that the enhanced senescence and apoptosis seen in the mammary tumor tissues of *Cdk4(R24C/R24C):MMTV-v-Ha-ras* mice could be due to an oncogenic stress-induced DNA-damage checkpoint response.



**Figure 4.6 Increased levels of  $\gamma$ -H2AX in the tumors of *Cdk4 R24C/R24C:MMTV-v-Ha-ras* mice.** (A) Formalin fixed paraffin embedded normal and tumor sections of the mammary glands of *Cdk4(+/+):MMTV-v-Ha-ras* and *Cdk4 (R24C/R24C):MMTV-v-Ha-ras* mice were subjected to immunohistochemical analysis using an antibody directed against  $\gamma$ -H2AX (100x). (B) Quantitation of  $\gamma$ -H2AX expression was determined using Bioquant software and analyzed using unpaired *t* test provided in Prism software.

### Key research accomplishments:

1. Our results indicate that Cdk4 expression is essential for ErbB2/Neu induced breast tumorigenesis
2. Cdk4 expression is not required for Wnt-1 induced breast tumorigenesis
3. Expression of Cdk4 is important for v-Ha-ras-induced mammary tumorigenesis.
4. Activating mutations in the Cdk4 gene such as the R24C do not accelerate v-Ha-ras-induced tumorigenesis.

### Reportable outcomes:

- A publication

“Reddy HK, Mettus RV, Rane SG, Graña X, Litvin J, Reddy EP. Cyclin-dependent kinase 4 expression is essential for neu-induced breast tumorigenesis. *Cancer Res.* 2005 Nov 15;65(22):10174-8.”

- Ph.D. degree

## Conclusions:

1. Cdk4 is required for Neu, Ras induced breast tumorigenesis.
2. Cdk4 is not required for Wnt-1 induced breast tumorigenesis.
3. Cdk4R24C mutation does not accelerate Ras induced breast tumorigenesis.

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# Cyclin-Dependent Kinase 4 Expression Is Essential for Neu-Induced Breast Tumorigenesis

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## Abstract

Previous work has shown that cyclin D1 expression is required for *neu*- and *ras*-induced, but not *wnt*- or *c-myc*-induced, breast tumorigenesis in mice. Although cyclin D1 binds and activates cyclin-dependent kinase 4 (Cdk4), thereby mediating activation of a program of E2F-dependent gene expression, it has been suggested that the oncogenic activities of cyclin D1 are independent of Cdk4. To determine whether Cdk4 expression is required for breast tumorigenesis in mice, we have generated compound mice ectopically expressing the *neu* or *wnt* oncogenes in the mammary glands of wild-type and *Cdk4*<sup>-/-</sup> mice. Our results show that Cdk4 expression is required for efficient *neu*-induced tumorigenesis but is dispensable for *wnt*-induced breast tumorigenesis. In contrast to results previously observed in the mammary glands of cyclin D1<sup>-/-</sup> virgin females, our results show defects in mammary gland development in *Cdk4*<sup>-/-</sup> virgin females, suggesting differences in compensatory mechanisms in the absence of either subunit of the cyclin D1/Cdk4 complex. These results suggest that drugs targeted to inhibit Cdk4 activities could be developed to specifically treat certain breast tumors as Cdk4 is not essential for viability. (Cancer Res 2005; 65(22): 10174-8)

## Introduction

A key response to growth factors in many cell types is the activation of cyclin-dependent kinase (Cdk) 4 or Cdk6 by members of the cyclin D family (D1, D2, and D3). D-type cyclins are expressed at low levels in a variety of quiescent cell types and their expression is stimulated by growth factors and mitogens (1–5). Approximately 50% of human mammary carcinomas express abnormally high levels of cyclin D1 (6–10), which is maintained throughout subsequent stages of breast cancer progression from *in situ* carcinoma to invasive carcinomas (9, 11, 12). Consistent with the oncogenic role of cyclin D1 in mammary epithelium, transgenic mice overexpressing cyclin D1 in their breast tissue have been found to develop mammary adenocarcinomas (13). Furthermore, loss of cyclin D1 was found to affect breast development (14, 15). More importantly, cyclin D1 null mutant mice were found to be resistant to breast cancers induced by the *neu* and *ras* oncogenes but remained fully sensitive to other oncogenic pathways driven by c-Myc or Wnt-1 (16). A requirement for D-type cyclins in cellular transformation

*in vitro* has also been shown using triple cyclin D knockout mouse embryonic fibroblasts, which are resistant to transformation by c-Myc or Ras in combination with dn-p53, E1A, or c-Myc (17). Similarly, *Cdk4* null mouse embryonic fibroblasts have been shown to be refractory to transformation by Ras and dn-p53 and, consistent with these data, the hyperactive *Cdk4R24C* allele cooperates with single oncogenes to transform mouse embryonic fibroblasts *in vitro* (18, 19). Taken together, these results suggest that the activity of D-type cyclin/Cdk complexes is required for fibroblast transformation. However, it has also been suggested that the oncogenic function of cyclin D1 is independent of its ability to activate Cdk4 and is perhaps linked to the direct effects of cyclin D1 in controlling the expression of a subset of genes that are co-up-regulated in human tumors with deregulated cyclin D1 (20).

Thus, whereas a role for cyclin D1 in breast cancer is well established, it is not known whether the oncogenic function of cyclin D1 requires Cdk4. To understand the role of Cdk4 *in vivo*, we have targeted the mouse *Cdk4* locus by homologous recombination in embryonic stem cells and generated a strain of mice that does not express *Cdk4* [*Cdk4(neo/neo)*; ref. 21]. Homozygous *Cdk4(neo/neo)* null mutant mice are viable and were found to be very resistant to carcinogen-induced cancers (data not shown). In this communication, we show that loss of *Cdk4* expression results in poor mammary gland development that is characterized by impaired ductal branching. In addition, we show that Cdk4 expression is essential for *neu*-induced breast tumor development; on the other hand, it is dispensable for *wnt*-induced breast tumor development.

## Materials and Methods

**Generation of *Cdk4(neo/neo)*/mouse mammary tumor virus transgenic mice.** To generate compound mice that express *neu* and *wnt-1* oncogenes in a Cdk4 null background, *Cdk4(neo/+)* mice were mated with mouse mammary tumor virus (MMTV)-*neu* and MMTV-*wnt-1* transgenic mice to generate *Cdk4(neo/+)*/MMTV-*neu* and *Cdk4(neo/+)*/MMTV-*wnt-1* mice, respectively. These mice were then intracrossed to generate *Cdk4(neo/neo)*/MMTV-*neu* and *Cdk4(neo/neo)*/MMTV-*wnt-1* transgenic mice.

**Whole-mount and histopathologic analysis of mammary glands.** The fourth inguinal mammary glands were dissected, spread onto a glass slide, and fixed with a mixture (1:3) of glacial acetic acid/ethanol, hydrated, stained with 0.2% carmine and 0.5% AlK(SO<sub>4</sub>)<sub>2</sub>, dehydrated in graded solutions of ethanol, and cleared in toluene and methyl salicylate as described previously (14). Carmine-stained or formalin-fixed mammary glands were also routinely processed for paraffin embedding and were stained with H&E.

**Protein analysis.** Mammary glands or tumors were homogenized in TNE lysis buffer and lysates were cleared by centrifugation. Protein, 50 to 100 µg, was resolved by SDS-PAGE and was transferred to nitrocellulose membranes. Immunoblots were probed with antibodies against HER2/ErbB2 (Cell Signaling Technology, Beverly, MA), Cdk4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Cdk6 (NeoMarkers, Fremont, CA), Cdk2 (Santa Cruz Biotechnology), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Abcam, Cambridge, MA), retinoblastoma (Rb; BD Biosciences, San Diego, CA), and phosphorylated Rb (pRb; Ser<sup>780</sup>; Cell Signaling Technology).

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## Results

**Cdk4 is required for proper development of mammary epithelium.** To gain an insight into the role of Cdk4 in breast development, we first examined the status of mammary epithelium in wild-type [*Cdk4*(+/+)] and *Cdk4*-deficient [*Cdk4*(*neo/neo*)] mice. Examination of H&E-stained mammary gland whole mounts derived from virgin female mammary glands at 14 to 17 weeks revealed striking differences in the extent of mammary gland ductal outgrowth in the two sets of mice (Fig. 1A and C). In *Cdk4*(*neo/neo*) mice, both ductal outgrowth and branching morphogenesis was considerably reduced when compared with their wild-type counterparts. In addition, an examination of the longitudinal sections of the mammary tissue sections also showed a distinctive reduction in the number of mammary ducts and a complete absence of alveoli (Fig. 1B and D). These observations suggest that loss of Cdk4 expression in breast epithelium results in a diminution of mammary gland ductal branching where alveolar segments were markedly fewer in number compared with the wild-type mammary gland.

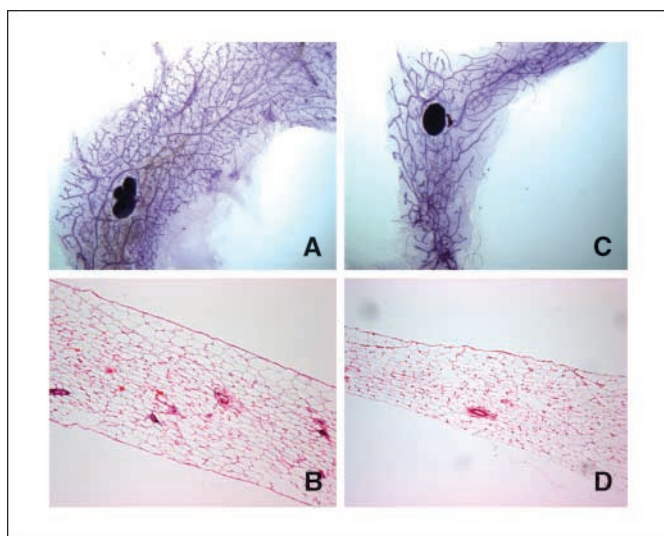
**Effect of loss of Cdk4 expression on *neu*- and *wnt*-mediated breast tumorigenesis.** To study the role of Cdk4 in *neu*- and *wnt*-induced breast tumorigenesis, *Cdk4*(*neo/neo*) mice were bred with MMTV-*neu* and MMTV-*wnt* transgenic mice to generate *Cdk4*(*neo/neo*):MMTV-*neu*, and *Cdk4*(*neo/neo*):MMTV-*wnt* mice, respectively (Fig. 2A). Whole-mount and histopathologic sections of the mammary glands derived from virgin adult mice (~14 weeks) from these different crosses (Fig. 2B and F) showed that *Cdk4*(+/+):MMTV-*neu* mice exhibit proliferative disturbances in the mammary epithelium as evidenced by the appearance of multiple hyperplastic and dysplastic nodules that infiltrate the mammary fat pad (Fig. 2B and F), which is in accordance with the published data (22). Similar examination of whole-mount and histopathologic sections of mammary tissue derived from *Cdk4*(*neo/neo*):MMTV-*neu* mice showed that the ductal outgrowth and branching morphogenesis was considerably reduced compared with *Cdk4*(+/+):MMTV-*neu*

mice with distinctive absence of any hyperplastic or dysplastic nodules that are characteristic of the latter group of mice (Fig. 2C and G). Histopathologic examination of these mammary glands also failed to show abnormal proliferative disturbances in the mammary epithelium of *Cdk4*(*neo/neo*):MMTV-*neu* mice (Fig. 2C and G). This does not seem to be due to lack of Neu expression, as equal levels of Neu protein was seen in both *Cdk4*(+/+):MMTV-*neu* and *Cdk4*(*neo/neo*):MMTV-*neu* mice (Fig. 2J). These results suggest that Cdk4 expression is essential for the appearance of MMTV-*neu*-induced proliferative disturbances that are seen in *Cdk4*(+/+):MMTV-*neu* mice.

In contrast to MMTV-*neu* mice, the mammary glands of virgin *Cdk4*(+/+):MMTV-*wnt* mice showed precocious lobuloalveolar development that resembles that of *Cdk4*(+/+) pregnant female mice (Fig. 2D and H), similar to previously reported observations (23). Histopathologic examination of these mammary glands revealed extensive appearance of hyperplastic alveolar nodules, which seem to be preneoplastic lesions (23). Similar examination of whole-mount and histopathologic sections of mammary tissue derived from *Cdk4*(*neo/neo*):MMTV-*wnt* mice showed that the ductal outgrowth and branching morphogenesis was unaltered compared with *Cdk4*(+/+):MMTV-*wnt* mice (Fig. 2E and I). Histopathologic examination of these mammary tissues again showed extensive appearance of hyperplastic alveolar nodules, similar to that seen with wild-type MMTV-*wnt* mice. These results indicate that MMTV-*wnt*-induced proliferative disturbances do not require Cdk4 expression.

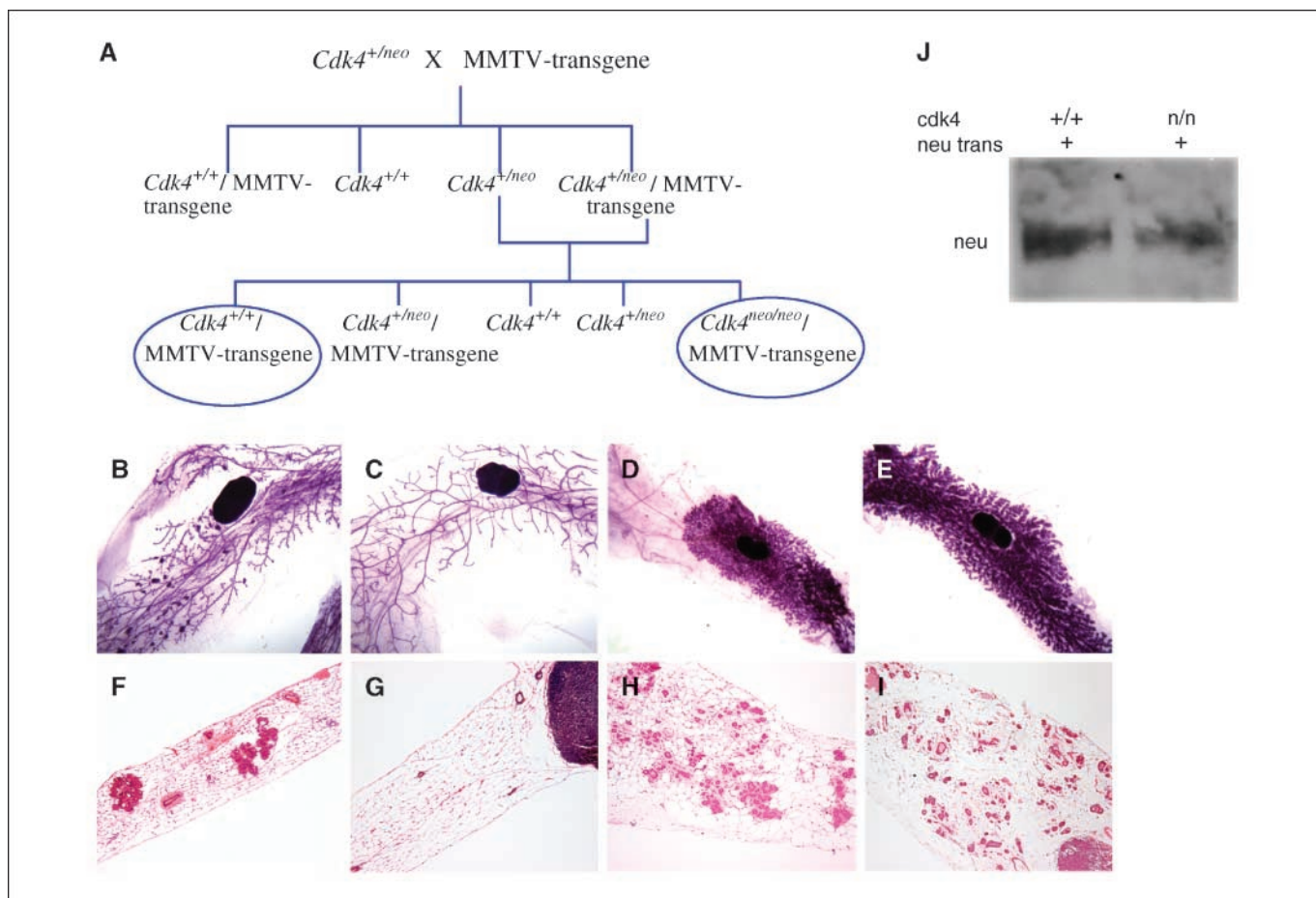
**Loss of expression of Cdk4 influences the incidence of mammary carcinomas.** It has been previously reported that MMTV-*neu*-induced breast carcinomas require the expression of cyclin D1, whereas those induced by MMTV-*wnt* do not require the expression of cyclin D1 (16). To determine whether Cdk4 plays a similar role in the development of breast carcinomas, we monitored the four groups of transgenic mice for the appearance of breast tumors. The results of this study presented in Fig. 3A show that ~97% of the *Cdk4*(+/+):MMTV-*neu* mice develop breast cancer between 28 to 75 weeks of age. The rest of the mice were found to develop salivary gland tumors. In sharp contrast, only ~14% of the *Cdk4*(*neo/neo*):MMTV-*neu* mice develop signs of breast cancer and this incidence was found to occur only after ~60 weeks of age; when these tumors arise, they were very small in size compared with their wild-type counterparts. Calculation of *P* values showed a highly significant increase in tumor frequency ( $P = 2.3 \times 10^{-6}$ ) for *Cdk4*(+/+):MMTV-*neu* mice as opposed to their *neo/neo* counterparts. These observations suggest that development of breast tumors in MMTV-*neu* transgenic mice requires normal expression of Cdk4.

In contrast to *Cdk4*(+/+):MMTV-*neu* mice, both *Cdk4*(+/+):MMTV-*wnt* mice and *Cdk4*(*neo/neo*):MMTV-*wnt* mice exhibited a rapid onset of breast tumors around 10 weeks of age and >90% of these mice developed breast tumors by the age of 30 weeks (Fig. 3B). Our studies also show that there was a slight delay in the development of breast tumors in *Cdk4*(*neo/neo*):MMTV-*wnt* mice compared with their wild-type counterparts. In contrast to the results observed for MMTV-*neu* mice, no significant difference in tumor frequency ( $P = 0.7264$ ) was observed between *Cdk4*(+/+):MMTV-*wnt*-1 and their *neo/neo* counterparts. The incidence of breast tumors was seen in both male and female mice as has been previously described (22). These observations show that Cdk4 expression is dispensable for MMTV-*wnt*-induced breast tumorigenesis.



**Figure 1.** Impaired mammary epithelial expansion in *Cdk4*(*neo/neo*) mice. The fourth inguinal mammary glands from *Cdk4*(+/+) (A) and *Cdk4*(*neo/neo*) (C) mice at 14 weeks of age were removed, fixed, and stained with carmine alum stain overnight at room temperature. Histologic sections of the fourth inguinal mammary glands from *Cdk4*(+/+) mice (B) and *Cdk4*(*neo/neo*) mice (D) were stained with H&E.





**Figure 2.** Loss of Cdk4 impairs MMTV-neu-induced breast epithelial cell proliferation and formation of preneoplastic nodules but not of the MMTV-wnt1-induced transformation. **A**, crosses done to produce the required transgenic mice. Whole mounts were made from the fourth inguinal mammary glands of  $Cdk4^{+/+}$ /MMTV-neu (**B**),  $Cdk4^{neo/neo}$ /MMTV-neu (**C**),  $Cdk4^{+/+}$ /MMTV-wnt-1 (**D**), and  $Cdk4^{neo/neo}$ /MMTV-wnt-1 (**E**) transgenic mice. **F**, **G**, **H**, and **I**, H&E-stained sections of the mammary glands shown in **B**, **C**, **D**, and **E** respectively. **J**, Western blot analysis of mammary tissue extracts derived from  $Cdk4^{neo/neo}$  and  $Cdk4^{+/+}$  mice. Each lane contains 100  $\mu$ g of protein. Western blot analysis was done with an anti-neu antibody.

The histopathologic sections of the tumors is given in Fig. 3C. These sections show that  $Cdk4^{+/+}$ :MMTV-neu tumors have a high density of epithelial cells, whereas the tumor sections of  $Cdk4^{neo/neo}$ :MMTV-neu mice show increased infiltration by connective tissue. MMTV-wnt tumors in a  $Cdk4^{+/+}$  or  $Cdk4^{neo/neo}$  background showed a similar phenotype with a high density of epithelial cells. Interestingly, these tumors show increased vasculature, suggesting that the Wnt pathway promotes angiogenesis, which might explain the very rapid growth of tumors in these mice.

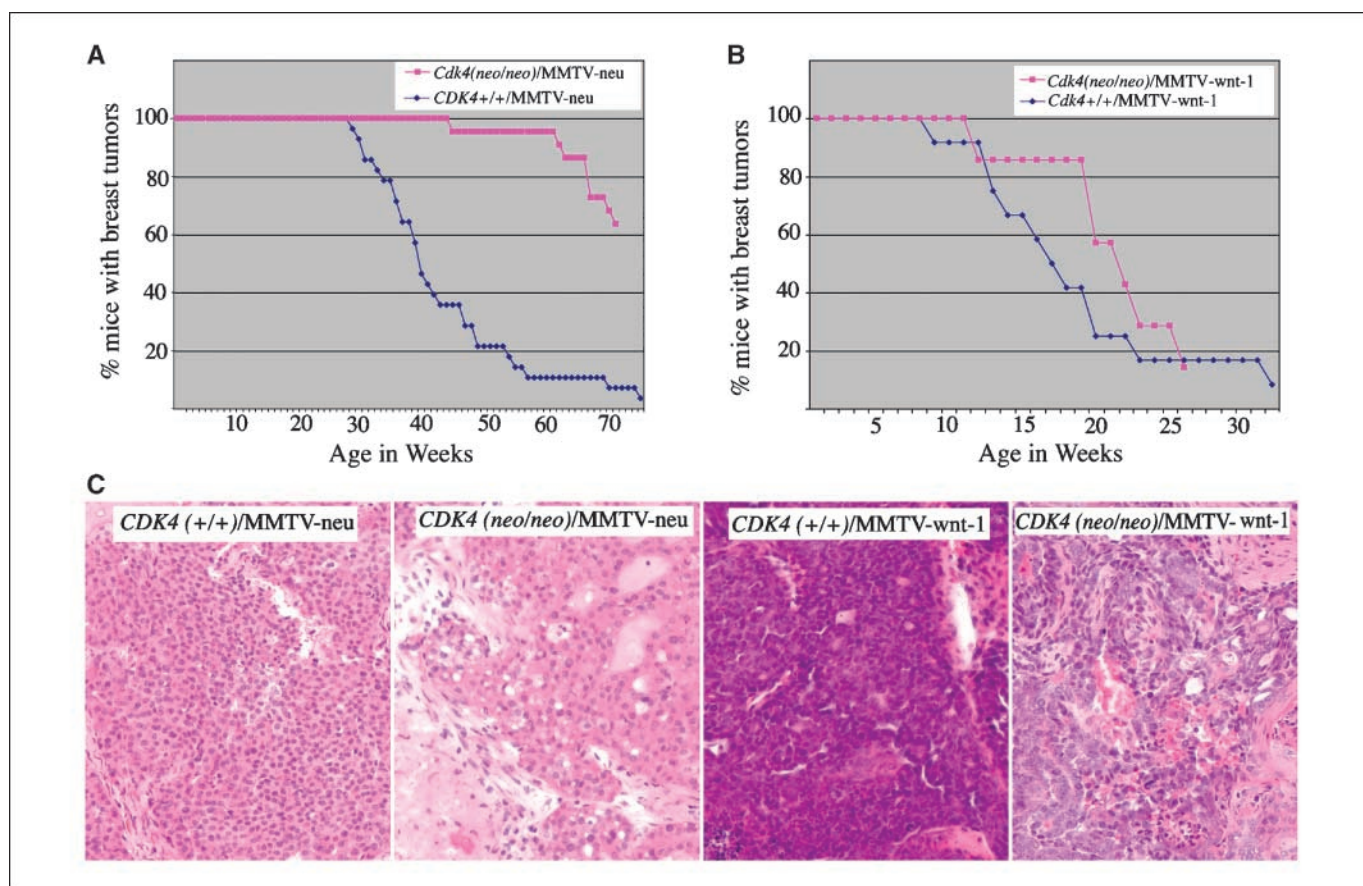
**Expression patterns of Cdk4, Cdk6, and Cdk2.** It has been previously shown that loss of *cyclin D1* results in a breast tumor phenotype similar to that described here for  $Cdk4^{neo/neo}$  mice. In the case of MMTV-wnt:cyclin D-/- mice, loss of *cyclin D1* seemed to be compensated by the overexpression of cyclin D2, which could drive the cell cycle progression (16). To understand the molecular basis for the development of breast tumors in MMTV-wnt mice in a  $Cdk4$  null background, we examined the expression patterns and kinase activities of Cdk6 and Cdk2 in all four of the genotypes studied here. The results presented in Fig. 4A show that in both  $Cdk4^{neo/neo}$ :MMTV-wnt and in  $Cdk4^{neo/neo}$ :MMTV-neu mice, the levels of Cdk4 were undetectable, whereas the levels of Cdk4 were pronounced in  $Cdk4^{+/+}$ :MMTV-neu and  $Cdk4^{+/+}$ :MMTV-wnt mice. In contrast, the levels of Cdk6 and Cdk2 were

approximately equal in all four genotypes. These results suggest that neither Cdk6 nor Cdk2 compensate for the loss of Cdk4 in MMTV-wnt transgenic mice on a  $Cdk4^{neo/neo}$  background. We next examined the expression levels and the phosphorylation status of Rb in  $Cdk4^{neo/neo}$ ,  $Cdk4^{+/+}$  as well as the MMTV-neu and MMTV-wnt transgenic mice crossed to the two Cdk4 backgrounds. Results of these experiments presented in Fig. 4B show that the level of pRb Ser<sup>780</sup> phosphorylation was low in  $Cdk4^{+/+}$  tissues but showed considerable elevation in those derived from both  $Cdk4^{+/+}$ :MMTV-neu and  $Cdk4^{+/+}$ :MMTV-wnt mice, which corresponds to the high levels of Cdk4, Cdk6, and Cdk2 activities seen in these tissues.

## Discussion

Our studies reported in this article suggest the importance of *Cdk4* in mammary gland development and tumorigenesis. Whole-mount analysis and histologic sections of  $Cdk4^{neo/neo}$  and  $Cdk4^{+/+}$  mice show that Cdk4 is required for proper ductal branching and lobuloalveolar development of virgin female mice. In contrast, the mammary glands of *cyclin D1*-/- virgin females have been reported to be identical to that of wild-type mice. This difference is likely due to the compensation by cyclins D2 and D3,





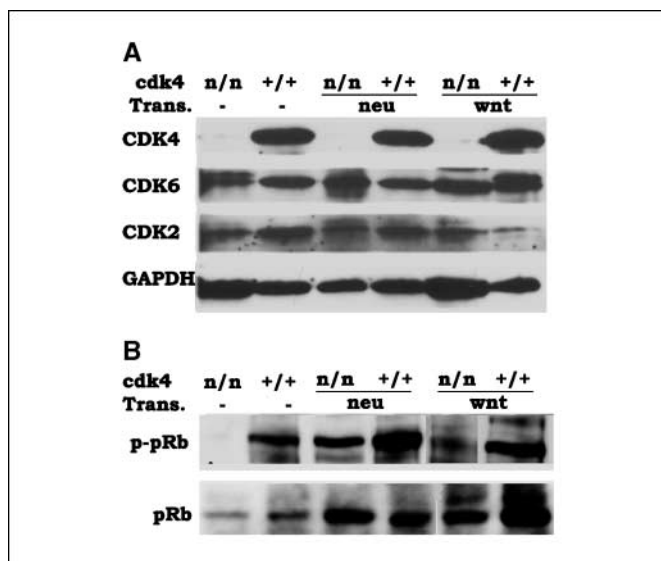
**Figure 3.** Loss of Cdk4 results in reduced and delayed tumor incidence in *Cdk4(neo/neo)/MMTV-neu* transgenic mice. **A**, tumor incidence in *Cdk4(neo/neo)/MMTV-neu* and *Cdk4(+/+)/MMTV-neu* mice over a period of 75 weeks. **B**, tumor incidence in *Cdk4(neo/neo)/MMTV-wnt-1* and *Cdk4(+/+)/MMTV-wnt-1* mice over a period of 31 weeks. **C**, histology of tumors in MMTV-*neu* and MMTV-*wnt* mice bred against *Cdk4(+/+)* or *Cdk4(neo/neo)* background. Tumor sections were stained with H&E and photographed at a magnification of  $\times 100$ .

which are slightly up-regulated in the mammary gland of *cyclin D1*<sup>-/-</sup> virgin females (16). Our results suggest that in the absence of Cdk4, there is no parallel compensation by other Cdk4s (Fig. 4, see below). Regardless of the lack of defects in the mammary gland of *cyclin D1*<sup>-/-</sup> virgin females, it has been shown that *cyclin D1*<sup>-/-</sup> mice fail to undergo full lobuloalveolar development during late stages of pregnancy (14, 15). It has also been shown that the *cyclin D1* null mice are prone to transformation induced by the *wnt-1* and *myc* oncogenes, but not to transformation induced by the *neu* and *ras* oncogenes (16). Recent studies on MMTV-*erbB2*-MMTV-*p16* double-transgenic mice showed that *erbB2*-mediated tumorigenesis is blocked by *p16* and that these double-transgenic mice develop rare tumors after a long delay (24). Because *Cdk4(neo/neo):MMTV-neu* mice showed decreased levels of ductal branching and lobuloalveolar development of the mammary glands when compared with that of *Cdk4(+/+):MMTV-neu* transgenic mice, we presume that Cdk4 is required for Neu-induced proliferative events that lead to ductal branching, lobuloalveolar development, and the development of hyperneoplastic alveolar nodules, and ultimately for the development of mammary tumors. We cannot rule out, however, that the observed defects in *Cdk4(neo/neo)* mammary gland development be the indirect result of hormonal signaling deficiencies as opposed to an epithelial cell autonomous defect.

The mammary glands of *Wnt-1* transgenic virgin mice undergo precocious lobuloalveolar development and resemble the mam-

mary glands of wild-type nontransgenic pregnant females. Our whole-mount and histologic studies of the mammary glands of *Cdk4(neo/neo):MMTV-wnt-1* and *Cdk4(+/+):MMTV-wnt-1* mice showed comparable lobuloalveolar development. This suggests that Cdk4 is not required for Wnt-1-induced ductal branching and lobuloalveolar development. The tumorigenesis studies conducted by us also show that both strains of mice are equally susceptible to Wnt-1-induced tumorigenesis, suggesting that Cdk4 is not required for this process. Thus, if the defect in mammary development observed in *Cdk4 neo/neo* females is not cell autonomous, then Wnt not only bypasses Cdk4 function, but also any conceivable defects in hormone signaling resulting from Cdk4 ablation. Our data also showed that the level of pRb phosphorylation on Ser<sup>780</sup> correlated with G<sub>1</sub> Cdk activities. We have also seen that phosphorylation of this site on pRb is highly increased in breast tumor tissues independently of Cdk4 phosphorylation status, suggesting that in highly proliferative tumors, this site could be phosphorylated by Cdk4s other than Cdk4 (data not shown).

Considering previous results indicating that Neu may act by inducing cyclin D1 expression and our results shown here that Cdk4 is required for *neu*-induced tumorigenesis, we propose that the cyclin D1/Cdk4 complex is required for *neu*-induced tumorigenesis. It has also been suggested that *wnt*- and *c-myc*-induced breast tumorigenesis communicate with the cell cycle machinery in breast epithelial cells through different targets. In this regard,



**Figure 4.** Cdk4, Cdk6, and Cdk2 expression and activity. **A**, Western blot analysis of the protein extracted from frozen mammary tissues of *Cdk4(neo/neo)*, *Cdk4(+/+)*, *Cdk4(neo/neo)/MMTV-neu*, *Cdk4(+/+)/MMTV-neu*, *Cdk4(neo/neo)/MMTV-wnt-1*, and *Cdk4(+/+)/MMTV-wnt-1* transgenic mice using antibodies against Cdk4, Cdk6, Cdk2, and GAPDH (loading control). Each lane contains 50  $\mu$ g of protein. **B**, expression of unphosphorylated Rb (*pRb*) and phosphorylated Rb (*p-pRb*) in mouse mammary extracts derived from (**A**). Each lane contains 50  $\mu$ g of protein. *n/n*, *neo/neo*.

cyclin D2 expression was found to be up-regulated in tumors induced by *wnt-1* and *c-myc*, but not *neu* or *ras* (16). Considering our data showing that Cdk4 is also dispensable for *Wnt*-induced tumorigenesis, and the lack of obvious compensation by other  $G_1$  Cdks, it is tempting to speculate that *Wnt* signals downstream of D-type cyclin/Cdk4 complexes. In summary, our data suggest that, at least in the case of *Neu*-induced tumorigenesis, a Cdk4 function is required. This requirement could be for Cdk4 kinase activity, or, alternatively, for the ability of the cyclin D/Cdk4 complex to sequester p27. Further studies are necessary to differentiate between these two possibilities.

These results also have important implications with respect to therapeutic modalities that might be effective in the treatment of breast cancers that are *neu*-positive. The importance of Cdk4 and cyclin D1 complex in the genesis of *Neu*-induced breast tumors suggests that small molecule inhibitors of Cdk4 kinase activity could be very effective in blocking the growth of these human breast tumors, which often represent the most aggressive forms of human breast cancer.

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